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AWARD NUMBER DAMD17-97-1-7050

TITLE: Cloning of Novel Oncogenes Involved in Human Breast Cancer

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REPORT DATE: June 1998

TYPE OF REPORT: Annual

PREPARED FOR: Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for public release;
distribution unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	June 1998	Annual (1 Jun 97 - 31 May 98)

4. TITLE AND SUBTITLE

Cloning of Novel Oncogenes Involved in Human Breast Cancer

5. FUNDING NUMBERS

DAMD17-97-1-7050

6. AUTHOR(S)

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7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research And Materiel Command
ATTN: MCMR-RMI-S
504 Scott Street
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 words)*

The genetic determinants which lie behind the development of breast cancer remain largely uncharacterized. In order to identify genes which may play a role in breast cancer we have begun a process of manufacturing cDNA expression libraries derived from human breast tumor cell lines in retroviral vectors. These libraries will be screened for their ability to induce a transformed phenotype in non-transformed or sub-transformed breast epithelial cells. The genes responsible for the transformation will be isolated with the goal of identifying important new markers for tumorigenesis which may serve as the targets for intervention or diagnosis.

14. SUBJECT TERMS

Breast Cancer Oncogenes, Retroviral vectors

15. NUMBER OF PAGES 8.

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

FOREWORD

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Table of Contents

5. INTRODUCTION

The multi-step nature of cancer means that multiple genetic lesions must occur for a normal cell to complete its transformation into a tumor cell (1,2). These lesions may occur in a variety of different combinations which vary in their probability from tissue to tissue. Whereas this has made the elucidation of the precise molecular basis of cancer exceedingly difficult, it also presents the opportunity of multiple targets for intervention or diagnosis. Consequently, great efforts have been made to identify and characterize the genetic determinants of Cancer.

Although considerable success has been achieved in cloning oncogenes and tumor suppressers, the size of the human genome and the failure of most of these genes to show any great epidemiological connection with human disease strongly suggests that there are other markers of critical importance which remain unidentified. This is particularly true of breast cancers.

Almost all experimental systems designed to detect oncogenes have essentially relied upon the ability of the gene to transform fibroblast cells(3,4,5). This is simply because of the technical ease of gene transfer in such systems. However, this approach fails to consider that the majority of human cancers arise from epithelial based tissue. As the transformation profile of fibroblast and epithelial cells does not seem to be identical (6), it is possible that genes which are important for epithelial cell transformation have been missed by these fibroblast based screens.

The main reason for not using epithelial cells in oncogene "hunting" experiments has been the relative difficulty in transferring DNA into epithelial cells relative to fibroblasts. However, retroviral vector technology has now advanced to the level where such experiments using epithelial cells as a recipient indicator cells for oncogenes is feasible. The basis of this proposal is an attempt to identify novel genes which promote the transformation of human breast epithelial cells by screening cDNA libraries derived from human epithelial breast tumor cell lines on breast epithelial cells.

6. BODY

a) Experimental Methods:

1. Construction of cDNA libraries was performed by isolating mRNA from 10^8 human breast tumor cell line cells using a Qiagen kit. A sample of the mRNA was examined visually by gel electrophoresis and ethidium bromide staining to confirm the quality.

2. cDNA was prepared from 5 ug of the mRNA using a BRL superscript kit. BstX1 adapters were synthesized, annealed and used instead of the supplied EcoR1 linkers. The ligated cDNA was first purified away from unligated adapters using the kit supplied sepharose columns. However, we found that this left significant contamination by the adapters. Consequently, we purified the ligated cDNA by preparative gel electrophoresis. The adapter DNA was then ligated overnight into BstX1 digested pCTV1 or pCTV3B vectors (4) to give both selectable and non-selectable libraries. The ligation was then purified by Phenol/chloroform extraction followed by ethanol precipitation.

3. The ligation was taken up in ddH_2O and added to 100 ul of electrocompetent bacteria which had been prepared as recommended by Biorad. The bacteria were then electroporated in a 0.2 mm cuvette at 2.5kV, 200 Ohms, 25 uF using a Biorad Genepulser

II apparatus. The bacteria were recovered for 1 hour and then plated in top agar on an LB bottom agar plate containing Tetracycline and Ampicillin. The plates were allowed to set and then incubated overnight at 37°. The number of colonies was scored. Reactions which gave less than 10^7 colonies per ug of vector were discarded. The surface colonies were then rinsed off and the remaining bacteria recovered by scrapping off the soft agar and centrifuging it through sterile glass wool. The recovered bacteria were aliquoted and flash frozen in 16% glycerol for long term storage.

4. To generate retroviral library plasmid DNA for transfection, a frozen aliquot of bacteria was thawed into 500 ml of L-B/Tetracyclin,Ampicillin and grown to an O.D. 600 of 1. The plasmid DNA was then purified from the bacteria using a Qiagen column. Several hundred ug of DNA were usually recovered.

5. Transfection of BOSC 23 retroviral packaging cells was performed by Calcium phosphate precipitation as well as by Lipofectamine (Bethesda Research Laboratories) and Fugene (Boehringer Mannheim). Growth medium was supplemented with 20 nM sodium butyrate after 48 hours and conditioned medium collected after a further 24 hours. The viral conditioned medium was passed through a 0.45 u filter before adding to the target cells and any excess was flash frozen for storage at -180°. The viral titre was estimated by transfecting the BOSC 23 cells with an equal quantity of pCTV3B vector (which contains the hygromycin resistance marker) and scoring for the ability of 1 ml of viral conditioned medium to induce Hygromycin B resistant colonies on 3T3 cells.

b) Assumptions:

This project assumes that there remain undiscovered genes which play an important role in the transformation of human breast epithelia. Furthermore, it assumes that at least some of these genes will confer a phenotype on untransformed breast epithelial cells *in vitro* which will be detectable. The project also assumes that it will be technically feasible to generate representative cDNA libraries from human tumor cell lines and to package them into infectious retrovirus with high efficiency.

c) Procedures:

The project is based upon a process of retroviral mediated gene transfer of breast cell derived expression libraries. This allows the assay of an enormous number of genetic elements for their ability to participate in the neoplastic conversion of breast epithelial cells. Technical procedures employed include standard molecular biology techniques, construction of cDNA libraries from human tumor cell lines, extensive tissue culture, transfection, viral infection, and PCR .

d) Results and Discussion

The first stage of the project involves generating cDNA from two human breast tumor cell lines (T-47D and SK-Br-3) and using this to make cDNA libraries in selectable (CTV3B) and non selectable (CTV1B) retroviral vectors. The Qiagen mRNA preparation kit and the BRL Superscript cDNA synthesis kit proved quite successful in generating the cDNA. However, we found that the BRL supplied sepharose column was not a reliable means for removing contaminating, unligated adapters from the cDNA and several libraries had to be remade with cDNA purified by gel electrophoresis.

The first library attempted was derived from the T47-D cell line using the CTV1B and CTV3B vectors. Electrocompetent MC1061 bacteria containing the supF system on the P3 plasmid were prepared. Using the Biorad Genepulser II system it was possible to

generate over 10^6 independent colonies from CTV1B transfections. Ten colonies were examined at random. All were found to contain inserts. CTV3B transfections gave lower efficiencies and we are attempting to improve this by using larger quantities of DNA. At this stage, the laboratory moved to the NCI. After re-establishing the Lab, we manufactured cDNA from the second cell line Sk-Br-3. We are currently calibrating the new electroporation system in order to generate the new libraries.

Once established at the NCI, we began to test our retroviral production system: BOSC 23 cells. Although these cells had previously been able to generate quite high titres of virus, we found that our cells were no longer doing so. After extensive testing of the transfection/infection/culture conditions we began the process of acquiring fresh stocks from the American Tissue and Cell Collection (ATCC). However, on the advice of the inventor of these cells (Dr. Gary Nolan, Stanford, CA we have instead recently acquired the next generation of virus producing cells, Phoenix cells. These cells have been designed to be both more stable in culture and to be able to exceed the viral production of BOSC 23 cells by an order of magnitude. The Phoenix cells should greatly enhance the probability of the success of the project. We are currently calibrating the Phoenix cell system against our indicator cell lines.

While in the process of library generation we have generated several variant cell lines for use as indicator cell lines. These cells will serve to identify transforming sequences in the libraries. Firstly we have infected C127 cells with weakly activated forms of the oncogenes Raf and also RhoA. Neither of these oncogenes alone is able to promote the transformation of the cells. However, by activating their respective signaling pathways we hope that these cells will be "primed " for transformation by co-operating sequences which alone would not produce a detectable phenotype. We have also determined the optimum conditions for transformation by the Ras oncogene of a second indicator cell line, nMuMG murine breast epithelial cells. Essentially these are similar to those developed for the C127 line where the cells are grown in 5% serum instead of 10% after transfection to reduce any background of pseudo-transformants. We intend to use both lines as recipients as they may be susceptible to transformation by different spectra of oncogenes.

We have also generated a non-transformed human breast epithelial cell line which can be infected by rodent host range (ecotropic) virus. This line, MCF-10AEco, was generated by infecting MCF-10A human breast epithelial cells with the cloned ecotropic receptor protein using a human host range (amphotropic) delivery system. This line retained all essential, non-transformed, characteristics of the parental cell line but was now susceptible to infection with ecotropic virus at a similar efficiency to that seen with NIH 3T3 cells. We intend to include this cell line in the library screen to reduce the chance of losing any human specific determinants during the screen.

e) Recommendations:

Due to two physical relocations of the laboratory, one within the UNC system followed by one involving a more permanent move to the National Cancer Institute in Washington, the work is running slightly behind schedule. I do not anticipate further disruptions and I expect to recover lost time and meet the set targets in the coming year. However, I am considering taking advantage of the option to request an extension of the timeline of the grant by one year as described in section 5.(6) c of the Grant Agreement.

7. Conclusions:

The generation of retroviral cDNA libraries at sufficiently high complexity from human breast tumor cell lines in the retroviral vectors is technically feasible. Furthermore, we have found that it is possible to render a human breast cell line susceptible to infection

by the viral libraries by simple transduction of the receptor for the ecotropic viral particles in which the library will be packaged. Thus it may be possible to perform the search for novel human oncogenes in human cells. We now have the reagents to commence the screening stage of the project.

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